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SO VETERINARY PARASITOLOGY, (MAY 1993) Vol. 47, No. 3-4, pp. 225-233.

SO Z PARASITENKD, (1983) 69 (1), 27-34.*

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PURIFICATION OF *CRYPTOSPORIDIUM* OOCYSTS AND SPOROZOITES BY CESIUM CHLORIDE AND PERCOLL GRADIENTS

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Abstract. The lack of an adequate system for the in vitro cultivation of *Cryptosporidium* spp. has forced researchers to work on infected feces or tissues. Molecular and immunological analyses of *Cryptosporidium* stages must be preceded by complex preparatory steps involving the concentration, storage, purification, excystation of oocysts, and purification of sporozoites. This paper describes two new procedures for the purification of *Cryptosporidium*. The first, consisting of pretreatment of oocysts with sodium hypochlorite followed by concentration using a Percoll gradient, is suitable for nucleic acid analyses. The second, a concentration of untreated oocysts using a Cesium chloride gradient, is suitable for biochemical and immunological studies, but requires "fresh" oocysts.

Current interest in *Cryptosporidium* has stemmed from its association with the acquired immune deficiency syndrome (AIDS),[1] and its recognition as an agent of neonatal diarrhea in calves.[2,3] Characterization of *Cryptosporidium* species would facilitate epidemiological studies in order to determine the source of human infections. A prerequisite for biochemical and immunological analyses is the availability of adequate amounts of purified *Cryptosporidium* stages. A new procedure for the purification of *Cryptosporidium* oocysts and sporozoites is presented.

MATERIALS AND METHODS

Oocysts were obtained from 2 naturally infected calves' feces, submitted to Manitoba's Provincial Veterinary Laboratory. The oocysts were identified by a Kinyoun acid fast stain.[4]

The oocyst-containing feces were stored for 6 months at 4°C in a 2.5% potassium dichromate solution,[5] and oocysts were concentrated by a modification of the dichromate sucrose flotation procedure of Willson and Acres.[6] Briefly, a solution consisting of 9 ml of 2.5% K2Cr207-preserved feces, 24 ml of sucrose (825 g in 500 ml distilled water) and 6 ml of distilled water was placed in a 50 ml screw-cap centrifuge tube, inverted 10 times and centrifuged at 800 × *g* for 10 min; the surface layer was washed with 0.85% saline and centrifuged at 1,200 × *g* for 5 min; washing was repeated 3 times, and oocysts were counted using a hemocytometer.

Concentrated oocysts were used to experimentally infect 2 newborn calves, aged 3 and 5 days. Each was tube-fed a total of 2 × 10^6 oocysts in 1 ml of saline added to 500 ml of milk. The feces of both calves became positive for *Cryptosporidium* on day 4 post-infection. Feces were collected daily, concentrated and preserved as described above, and used for the purification studies. Some of these oocysts were used as antigen for an indirect fluorescent antibody (IFA) test.

Purification of oocysts

An attempt was made to purify oocysts using the following published procedures: the petri dish method by Hammond et al.[7] for Coccidia; the 5.25% sodium hypochlorite solution, recommended by Wagenbach et al.[8] for Coccidia; and the saturated sodium chloride procedure of Smith and Herrick[9] for Coccidia.

A new method for the purification of oocysts was developed combining two previously reported procedures which were modified and used in tandem. The first step is a modification of Wagenbach et al.,[8] and the second step is an ad-

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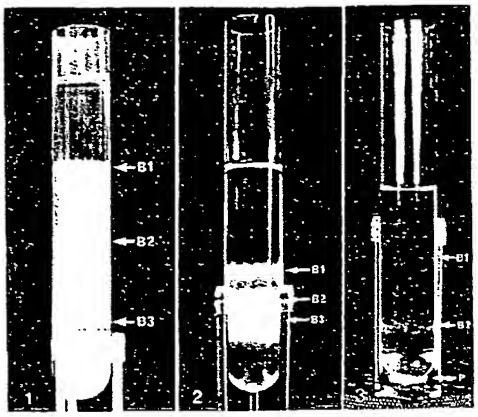

FIGURES 1–3. 1. Percoll gradient for the separation of sodium hypochlorite-treated oocysts. 2. Cesium chloride gradient for the separation of oocysts. 3. Cesium chloride gradient for the separation of sporozoites.

aptation of the Percoll procedure used by Fernando et al.[10] for the purification of *Eimeria* merozoites. Briefly, preserved oocysts were pretreated with 3% sodium hypochlorite by keeping the solution in an ice bath for 20 min then washing it with phosphate buffered saline (PBS), pH 7.2. The gradient of Percoll (Pharmacia, Quebec, Canada) consisted of 3 ml of stock (9 volumes of Percoll and 1 volume of 8.5% NaCl), 4 ml of 70% stock Percoll diluted with 0.85% NaCl, 4 ml of 50% stock Percoll diluted with 0.85% NaCl. Four ml of the oocyst suspension in PBS, pH 7.2, containing 23 × 10^6 oocysts, was layered over the gradient in 16 × 102 mm Ultra-Clear® tubes (Beckman Instruments, Inc.) and centrifuged at 16,000 × *g* for 10 min at 4°C. Three distinct bands were formed and removed separately, and washed 3 times with PBS, pH 7.2 (Fig. 1). At each stage of the sodium hypochlorite treatment and Percoll separation, aliquots were examined for bacterial contamination by inoculation of blood agar plates. In addition, 3 smears were prepared and stained by Kinyoun and Giemsa,[4] as well as Gram staining.[11] Proof of viability of oocysts was obtained by excystation and observing the motility of sporozoites microscopically. Some of these treated oocysts were used as a source of antigen for an IFA test.

A Cesium chloride gradient,[12] was prepared for the purification of oocysts not pretreated with sodium hypochlorite. Briefly, fecal oocysts were concentrated by the modified procedure of Willson and Acres,[6] washed twice with 0.85% saline and once with 50 mM Tris and 10 mM EDTA, and counted. One ml of oocyst suspension in 50 mM Tris and 10 mM EDTA containing 1 × 10^8 oocysts was layered on top of a CsCl gradient. The latter was prepared in 16 × 102 mm tubes containing CsCl in 50 mM Tris and 10 mM EDTA; the density was 1.40 g/ml in the bottom 3 ml, 1.10 g/ml in the middle 3 ml of the gradient and 1.05 g/ml in the top 3 ml. The CsCl gradient was centrifuged at 16,000 × *g* for 60 min at 4°C. Three bands were formed (Fig. 2); each was removed separately and dialyzed against 10 mM Tris and 1 mM EDTA. Each band was checked for bacterial contamination and 3 smears were prepared for staining as described above. Some of these oocysts were used as antigen for an IFA test, as well as for an ELISA.

Purification of sporozoites

For the excystation of oocysts, the method of Current et al.[13] was modified by washing 1:75% sodium hypochlorite-pretreated oocysts with Hanks Balanced Salt Solution (HBSS), free of Ca^{++} and Mg^{++}, as recommended by Reduker and Speer.[14]

For the purification of sporozoites using Cesium chloride gradient, various concentrations and centrifugation times were tried. The most successful combination was obtained by layering 1 ml of excysted oocysts in HBSS, pH 7.2, containing approximately 140 × 10^6 sporozoites over a gradient consisting of four 2-ml layers of CsCl with densities of 1.3, 1.2, 1.1, and 1.05 g/ml, respectively, from bottom to top. The gradient was centrifuged at 16,000 × *g* for 3 hr at 4°C. Two bands and a distinct pellet were formed (Fig. 3).

RESULTS

Of the five procedures used for the purification of oocysts only the two methods described here were of practical value. Of the 1 × 10^6 oocysts used in the glass petri dish experiment, only 10% were recovered. More than 95% of the 9 × 10^6 oocysts treated with 5.25% sodium hypochlorite and of the 7 × 10^6 oocysts concentrated by saturated sodium chloride appear to have excysted since intact oocysts could hardly be seen when examined microscopically. Each of the above procedures was repeated 3 times with identical results.

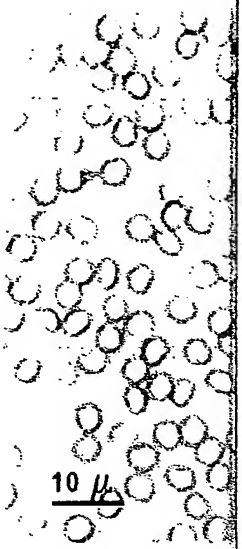

FIGURE 4. Unstained oocysts.

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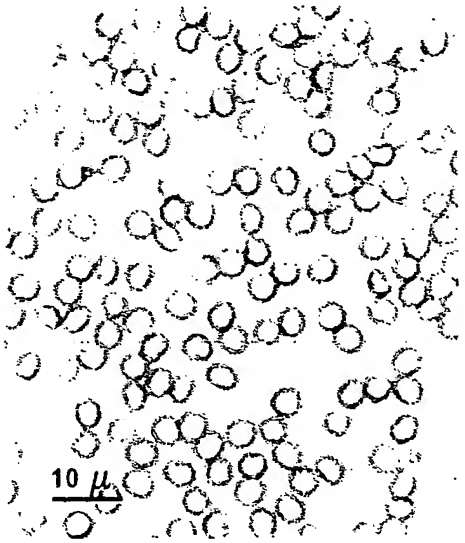

FIGURE 4. Unstained Cesium chloride-purified oocysts.

A 3% sodium hypochlorite pretreatment of oocysts followed by a Percoll gradient resulted in the recovery of 75% of the oocysts. Bands 2 and 3 (Fig. 1) were examined microscopically and shown to contain clean oocysts with no debris. After incubation at 37°C for 1 hr, microscopic examination showed motile excysted sporozoites indicating that the sodium hypochlorite-treated oocysts were viable. Tests for bacterial contamination were all negative. Direct smears and smears stained by Kinyoun acid fast and Giemsa showed a good concentration of oocysts. These oocysts demonstrated a >75% reduction in fluorescence when used as antigen for an IFA.

Whenever Percoll gradients were used on oocysts not previously treated with sodium hypochlorite, clean oocysts were not recovered from any of the bands; we attempted to change the length of centrifugation from 10 to 20 to 30 and 60 min without any success. As well, on three occasions we were unable to reproduce Waldman et al.'s findings.[15]

In the Cesium chloride gradient 3 bands and a distinct pellet could be recognized. Very clean viable oocysts, free of bacterial contamination were recovered from band 1 (Fig. 2). Lack of debris and excysted sporozoites were demonstrated microscopically. Furthermore oocysts from band 1 were successfully used to infect 1 newborn calf. Direct smears, Giemsa, and Kinyoun acid fast stained smears showed a good concentration of oocysts (Fig. 4). Approximately 94% of the oocysts were recovered by this procedure; they were suitable as source of antigen for IFA and ELISA and were used to raise hyperimmune antisera in rabbits. This 94% recovery was reported only when the oocysts used had been stored for <60 days. Recovery was reduced to 17.5% when oocysts were used after 10 months of storage and dropped to 5% after storage for one year.

The Cesium chloride gradient used for the purification of sporozoites showed 2 bands. Pure viable sporozoites were recovered mainly from band 2, while intact oocysts were found in the pellet (Fig. 3), as shown by microscopic examination.

DISCUSSION

Percoll gradient can be used for the concentration of sodium hypochlorite-treated oocysts provided all centrifugations are performed at 4°C to avoid excystation. Varying the centrifugation time from 10 to 60 min did not improve the concentration of untreated oocysts. We could not confirm the report of Waldman et al.[15] on the purification of oocysts yielding a concentration of 1×10^5/ml after 10 min centrifugation at $250 \times g$, using a Percoll discontinuous density gradient. Sodium hypochlorite-treated oocysts are suitable for nucleic acid and other biochemical analyses and for studies on sporozoites; however, they cannot be used for immunological studies, probably due to changes in the oocyst wall caused by the pretreatment. The reduction in fluorescence, as well as preliminary work using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), appear to confirm this hypothesis.

The Cesium chloride gradients produce oocysts and sporozoites that are suitable for immunological and biochemical studies. However to recover 94% of the oocysts, the suspension must be fresh. Methods for the cryopreservation of concentrated sporozoites are needed.

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CHRISTINE
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H. CLIFF
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